Department of

Oral and Maxillofacial Pathology and Oral Microbiology

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EVALUATION AND CORRELATION OF ANGIOGENIC AND MYOFIBROBLASTIC EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA - A CLINICOPATHOLOGICAL AND AN IMMUNOHISTOCHEMICAL STUDY

SYNOPSIS FOR REGISTRATION

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INTRODUCTION:

Head and neck cancers are the sixth most common cancer worldwide, resulting in more than 700,000 newly diagnosed cases in 2020 and over 400,000 deaths. Most head and neck cancers are squamous cell carcinomas, the most common being oral squamous cell carcinoma (OSCC) originating from the oral keratinocytes.[1] In South Asia, oral carcinoma is the second most common malignancy among males and females. One of the major global risk factors for carcinoma is smokeless tobacco and betel nut, which is consumed by 20% of the world population.[1]

OSCC patients exhibit significant symptom burden; primary treatment involves surgical resection, which is associated with high morbidity. Most OSCC patients are also treated with post-operative radiotherapy (PORT) and/or chemotherapy. These treatment modalities have an adverse impact on quality of life. There have been limited advances in OSCC management, with overall survival (OS) improving by only 5% in the last 20 years. Furthermore, OSCC prognostication and treatment selection still rely heavily on the tumor-node-metastasis (TNM) stagingsystem[2] and Broader`s classification for histological grading; where tumour biology has limited impact on treatment decisions. The most important prognostic factor is the presence or absence of metastases. Invasion and metastasis involve epithelial to mesenchymal transition (EMT). EMT is activated during development and wound healing, however, aberrant activation is associated with cancer progression.[5] The major role in gross and aggressive behavior of various pathological grades of OSCC is laid by tumoral stroma. Stromal reactions in cancer have a vital diagnostic and prognostic significance. The local aggregation of connective tissue cells and extracellular matrix characterizes epithelial tumors; this phenomenon has been labelled as the stromal reaction. Cells of the stroma have the capability to join actively in the progression of tumor, which in turn allows invasion and metastasis.[1]

Hence, identifying improved targeted treatments and prognostic markers are major priorities in OSCC. Identification of novel oncogenic drivers may permit more precise

treatment selection and reduce treatment-related morbidity in low-risk patients while improving the management of high-risk patients.[2]

CD34 is a transmembrane phosphoglycoprotein, first identified in 1984 on hematopoietic stem and progenitor cells, predominantly regarded as a marker of hematopoietic stem cells (HSC) and hematopoietic progenitor cells. However, CD34 is now also established as a marker of several other nonhematopoietic cell types, including vascular endothelial progenitors and embryonic fibroblasts. CD34 expression has also been demonstrated on several other cell types, including multipotent mesenchymal stromal cells (MSC), interstitial dendritic cells, and epithelial progenitor cells. Furthermore, CD34 expression may also be found in a variety of cancer stem cells. Nowadays, the molecular functions of this protein have been involved in a variety of cellular functions, such as enhancing proliferation and blocking cell differentiation, enhanced lymphocyte adhesion, and cell morphogenesis; yet, a complete understanding of this transmembrane protein, including its developmental origins, its stem cell connections, and other functions, is yet to be achieved.[7,8]

Normal epithelial cells are identified based on their histomorphological features and their immunohistochemical profile, where they express cytokeratins and absence of mesenchymal markers like α-SMA. Normal stroma contains an array of cells, the most abundant of which are the fibroblasts. Fibroblasts' main function is to provide support to the surrounding structures through the production of several ECM components. Myofibroblasts, however, are less abundant and are mainly located intra-orally in the walls of the blood vessels. They are recognized by α-SMA expression.[5]Alpha-smooth muscle actin antibody(α-SMA) labels smooth muscle cells, myofibroblasts(MF) and myoepithelial cells. For the identification of myofibroblasts, the commonly used marker is smooth muscle actin, which also helps in monitoring its behavior. An increase in the stromal MF is evaluated by the immunoreactivity of α-SMA in OSCC and has been linked with poor prognosis. The expression of alpha smooth muscle actin in the stroma of fibroblasts could be used as the index of OSCC in its supplemental diagnosis and judgment of severity.[1]

Studies have shown that normal mucosal stroma and squamous intraepithelial lesions (SILs) enclosed CD34-positive cells that were scattered, but there was an absence of α-SMA positive myofibroblasts. On the contrary, the stromal cells of SCC showed α-SMA positive myofibroblasts. The appearance of α-SMA positive myofibroblasts and the disappearance of CD34-positive stromal cells are linked with the conversion of squamous intraepithelial lesions (SILs) to SCC. The complete disappearance of CD34positive cells in the stroma in SCC indicates that they could have been converted into myofibroblasts. The appearance of CD34positiveendothelial cells plays a vital role in understanding the process of angiogenesis in oral cancer and pre-cancer.[1]

It is well understood that the activity that is coordinated between epithelial cells and their stroma is central in regulating growth and differentiation in normal as well as pathological conditions. Through the progression of the tumor, the stroma imitates disturbed interactions between the neoplastic population and its surroundings. Successful immunotherapy against cancer should essentially include complementary treatments against these tumor-associated fibroblasts. Although studies includingα-SMA markers among OSCC patients have been reported, rarely any study involving CD34 marker in OSCC cases have been reported. Also, there are no studies pertaining to follow-up of the patients to establish the above markers as definite prognostic markers.

Therefore, the purpose of the current study is to evaluate the expression of CD34andα-SMA and to establish a correlation of these biomarkers (CD34 andα-SMA)in relation to the clinical progression and grading of OSCC along with the treatment plan followed in order to establish a definite prognosis thus demonstrating the role of CD34 and α-SMA as prognostic markers in oral squamous cell carcinoma, therefore, confirming their diagnostic and prognostic significance and association with tumor differentiation. The study aims to prove a benchmark for further research giving away a plethora of insight into the field biomarkers of OSCC.

LITERATUREREVIEW:

1. **Arakelian L, Lion J, Churlaud G, Bargui R, Thierry B, Mutabazi E, et al. (2023)** did the analysis of CD34 taking in view expression and function and comparing high CD34 and low CD34 endothelial cell subpopulations. It was observed through transcriptomic analysis that CD34 gene and protein expressions are overtly correlated, and, CD34 high cells are less proliferative but highly expressive of IL-33 and Angiopoietin 2, as compared to CD34 low cells. ELISA confirmed higher secretion levels of IL-33 and Angiopoietin 2 by CD34 high HUVECs. When endothelial cells interacted with peripheral blood mononuclear cells, CD34 high endothelial cells showed stronger proliferation of regulatory T lymphocytes (Tregs) as compared to CD34 low cells while other CD4+- T cell subsets had shown equivalent expansion. The study results threw light on the fact that CD34 expression by endothelial cells in-vitro is associated with their ability to proliferate and has an immunogenic ability that favours the tolerogenic response.

2. **Taherkhani A, Dehto SS, Jamshidi S, Shojaei S. (2022)** The present study aimed to identify potential markers, including microRNAs (miRNAs) and genes, significantly involved in the etiology of early-stage OSCC. Additionally, the main OSCC's dysregulated Gene Ontology annotations and significant signaling pathways were identified. The dataset GSE45238 underwent multivariate statistical analysis in order to distinguish primary OSCC tissues from healthy oral epithelium. In patients with head and neck squamous cell carcinoma (HNSCC), upregulation of *CALM1*, *CYCS*, *THBS1*, *MYC*, *GATA6*, and *SPRED3* was strongly associated with a poor prognosis. In HNSCC patients, overexpression of *PIK3R3*, *GIGYF1*, and *BCL2L11* was substantially correlated with a good prognosis. Besides, “proteoglycans in cancer” was the most significant pathway enriched in the primary OSCC. The study results revealed more possible mechanisms mediating primary OSCC and may be useful in the prognosis of the patients with early-stage OSCC.

3. **Yasin MM, Abbas Z, Hafeez A.(2022)** conducted a study to find a correlation of histopathological pattern to the tumor site and habits as an outcome of OSCC. This cross-sectional study was conducted in Karachi, Pakistan. A total of 100 known cases of an oral squamous cell carcinoma were diagnosed with the help of biopsy reports and were examined for histopathologic features, site of the lesion, and risk habits. It was seen that 48 years was the mean age at the time of diagnosis with a distribution of 61% men and 39% women. The frequently affected site was buccal mucosa and the prime risk habit was gutka followed by betel quid. Histologically, the degree of differentiation shows that moderately differentiated OSCC was most commonly present, while the most prevalent histopathological pattern was spindle cell carcinoma. The statistical relation between lesion site and tobacco habits was found to be significant with a *p* value ($p = 0.01$). Thus concluding that rate of oral squamous cell carcinoma is higher in males than females with a mean age at the time of diagnosis being less than 50 years. Frequently placing gutka in the buccal vestibule against buccal mucosa is responsible to make buccal mucosa the most common tumor site.

4. **Rahman A, Alqahtani A, Aldhafferi N, Nasir MU, Khan MF, Khan MA, et al. (2022)**proposed model of transfer learning model using AlexNet in the convolutional neural network to extract rank features from oral squamous cell carcinoma (OSCC) biopsy images to train the model. Simulation results had shown that the proposed model achieved higher classification accuracy 97.66% and 90.06% of training and testing, respectively.

5. **Hsieh Y-P, Wu Y-H, Cheng S-M, Lin F-K, Hwang D-Y, Jiang S-S, et al. (2022)** conducted a study with the aim to verify the mechanisms underlying OSCC carcinogenesis with C. albicans infection and identify the biomarker for the early detection of OSCC and as the treatment target. The single-cell RNA sequencing analysis (scRNA-seq) was performed to explore the cell subtypes in normal oral mucosa, OPMD, and OSCC tissues. The cell composition changes and oncogenic mechanisms underlying OSCC carcinogenesis with C. albicans infection were investigated. Thus, the

detailed mechanism underlying OSCC carcinogenesis with C. albicans infection was determined and identified the treatment biomarker with potential precision medicine applications.

6. **Guo L, Mi J, Zhang H, Gao J, Zhang S, Li L, et al. (2022)**isolated cells from the normal dermal tissue and the wound tissue of mouse with full-thickness skin wound, and human scar tissue sections and α-SMA immunohistochemical staining and H and E staining was done. The ratio of factor VII or CD31/α-SMA double-positive cells in factor VII-positive cells was assessed in the isolated cells and in scar tissues. It was found that approximately 27–60% of ECs coexpressed VII factor and α-SMA in the isolated cells from the wound tissues of mice, which was significantly higher than that of normal dermal tissue cells. Accordingly, the number of CD31/α-SMA double-positive cells in mouse wound tissue sections was also significantly more than that in normal dermal tissue sections. In scar tissues, in addition to high-density microvessels, a large number of proliferative ECs in scar stroma and CD31/α-SMA double-positive cells were also found. Approximately 46.82 to 84.11% of ECs and 68.77 to 95.25% of myofibroblasts coexpressed VII factor and α-SMA, and these two values in hypertrophic scars were significantly higher than those in keloids. Thus confirming that ECs might contribute to the emergence of myofibroblasts in the wound and scar tissue via the process of EndMT.

7. **Oya K, Kokomoto K, Nozaki K, Toyosawa S (2022)** conducted a study with an aim to investigate AI ability to judge oral SCC in a novel training method considering cellular and structural atypia and their suitability. 90 digitized histological whole-slide images from randomly selected patients with tongue SCC who attended a dental hospital were examined. Results suggested that AI focused on both cellular and structural atypia of SCC, and tended to focus on the region surrounding the basal layer.

8. **Monteiro R, Hallikeri K, Sudhakaran A. (2021)** conducted a study on overall, 10 normal mucosa, 30 oral submucous fibrosis (OSF) and 30 oral squamous cell carcinoma (OSCC) with OSF and stained

immunohistochemically with phosphatase and tensin homologue (PTEN) and alpha-smooth muscle actin (α-SMA) with an aim to find the diagnostic role and correlation between phosphatase and tensin homologue and alpha-smooth muscle actin in oral submucous fibrosis and oral squamous cell carcinoma with concomitant oral submucous fibrosis. A significant decrease in nuclear PTEN positivity ($P < 0.001$) and a gradual increase in α-SMA cytoplasmic expression was noted. Thus it was concluded that, there exists statistically significant weak inverse correlation existed between PTEN and α-SMA.

9. **Caruntu A, Moraru L, Lupu M, Vasilescu F, Dumitrescu M, Cioplea M, et al. (2021)** conducted a study where evaluation of the prognostic character of tumor-infiltrating immune cells CD4+, CD8+ and CD56+ in oral squamous cell carcinoma (OSCC) was done. 90 OSCC patients were evaluated for densities of CD4+, CD8+ and CD56+ in two distinct compartments, intratumor and invasion front. CD4+ and CD8+ lymphocytes had significant differences between the tumor compartments. patients with high densities of intratumor CD8+ lymphocytes ($p = 0.0086$), CD8+ lymphocytes at the front of invasion ($p = 0.0011$) and for intratumor CD56+ cells ($p = 0.0016$) had shown an improved outcome (OS). Multivariate analysis proved the independent prognostic role of CD8+ at the front of invasion (OR = 3.75, CI95% 1.17–12.35, $p = 0.026$) and for intratumor CD56+ cells (OR = 3.669,CI 95 % 1.09–15.37, $p = 0.035$). Therefore, concluding that Tumor-infiltrating CD8+ lymphocytes at the front of invasion and CD56+ in the intratumor compartment display predictive traits in OSCC.

10. **Alabi RO, Bello IO, Youssef O, Elmusrati M, Mäkitie AA, Almangush A. (2021)** Improvement in prognostic evaluation of oral squamous cell carcinoma (OSCC) will immensely benefit the clinical management of oral cancer patients. Keeping this view in mind the study was conducted using five different databases—PubMed, Scopus, Ovid Medline, Web of Science, and Institute of Electrical and Electronic Engineers (IEEE). There were 34 studies that have used deep machine learning for the prognostication of

OSCC. The accumulated evidence indicated that deep machine learning models have great potential in the prognostication of OSCC.

11. **Tampakis A, Weixler B, Rast S, Tampaki E-C, Cremonesi E, Kancherla V, et al. (2021)**. Conducted a study with an intention to evaluate Nestin (a class VI intermediate filament protein of the cytoskeleton), and CD34, (a transmembrane phosphoglycoprotein, markers of progenitor cells) expression and clinical significance in colorectal cancer. 599 patients with colorectal cancer were analyzed by immunohistochemistry with a clinically annotated tissue microarray. Also, nestin and CD34 correlations with HIF-1a and a panel of cytokines and chemokines were evaluated using quantitative reverse transcription PCR and the Cancer Genome Atlas dataset. It was found that expression of nestin and CD34 was found only in the tumor stroma. Patients showing high expression of nestin and CD34 confirmed higher rates of T1 and T2 tumors (p¼.020), lower vascular invasion (p<.001) and improved 5-year overall survival (65%; 95% CI ¼ 55–73 vs 45%; 95% CI ¼ 37–53) after adjusting for clinicopathological characteristics (HR: 0.67; 95% CI ¼ 0.46–0.96). A moderate to strong correlation (r¼0.37–0.78, p<.03) of nestin and CD34 was demonstrated for the following markers; HIF-1a, CD4, CD8, FOXP3, IRF1, GATA3, CCL2, CCL3, CXCL12 and CCL21. Therefore, concluding that total expression of nestin and CD34 expression is associated with better overall survival possibly by modulating a favorable immune response.

12. **Lang K, Baur M, Held T, Shafie RE, Moratin J, Freudlsperger C, et al. (2021)** retrospectively examined 49 patients with non-metastatic primary unresectable OCC treated with definitive RT between 2000 and 2019. The majority of patients (63.3%) were treated with definitive chemoradiotherapy while 26.5% were given single-agent cetuximab weekly simultaneous to definitive RT. Five patients were treated with definitive RT alone because of limited disease and no nodal involvement. Results showed that median follow-up was 73 months (range, 6–236 months), median progression free survival (PFS) was 42 months (range, 2–157 months), median local disease-free survival (LDFS) was 44 months (range, 2–157 months) and

median overall survival (OS) from the time of RT initiation was 52 months (range, 5–236 months). There were 65.3% locoregional failures, 84.4% local and 15.6% distant metastasis. The majority of patients with local failure presented with American Joint Committee on Cancer (AJCC) Stage III–IV disease (59.2%). The 5-year Kaplan-Meier estimates for OS (III–IV *vs*. I–II) was 22.8% *vs*. 54.2 % (p = 0.03, HR 2.090, 1.1–4.2). Patients who were treated with systemic therapy had a significant better 5-year overall survival compared to those with RT alone (43.9% *vs*. 23.1%, p = 0.05, 1.0–4.1). RT with doses less than 70 Gy (p = 0.046, HR 2.1 (1.0–4.5) was associated with worse overall survival. Mucositis was the most common ≥ grade 3 acute toxicity and occurred in 19 patients (39%). Incidences of chronic toxicities were loss of taste, trismus, osteoradionecrosis and xerostomia. Thus concluding definitive RT with or without concurrent systemic agents in patients with unresectable OCC resulted in an eloquent rate of locoregional control and good overall survival rates and is currently the best available treatment option in this patient collective.

13. **Maqsood A. Ali, Zaffar Z, Mokeem S., Mokeem S.S, Ahmed N, Al-Hamoudi N, Vohra F, Javed F, Abduljabbar T et al. (2020)** conducted a study on 80 cases to analyze the expression of CD34 and alpha smooth muscle actin in OSCC, to establish their significance in diagnosis and prognosis. Immunohistochemistry was performed using antibodies against CD34 and α-SMA The chi-square and Pearson correlation coefficient (r) tests were applied for data analysis. Eighty patients with fifty males (62.5%) and thirty females (37.5%) were evaluated. Buccal mucosa was the most common site for OSCC lesions [36 (45%)]; 47.5% of lesions were moderately differentiated and 33.8% were well-differentiated lesions. Invasion of tumor vessels was observed in 35% of specimens. A significant association was seen between CD34 expression and histological grading of OSCC ($p < 0.002$). Among all poorly differentiated OSCC specimens, expression of CD 34 was low and α-SMA was high. They concluded that CD 34 is a critical prognostic factor in OSCC diagnosis and increased α-SMA positive myofibroblasts may indicate aggressive OSCC behavior.

14. **Radtke S, Pande D, Cui M, Perez AM, Chan YY, Enstrom M, Schmuck S, et at. (2020)** Hematopoietic stem cell (HSC) gene therapy has the potential to cure many genetic, malignant, and infectious diseases. The study has shown that in a nonhuman primate gene therapy and transplantation model the CD34+CD90+ cell fraction were exclusively responsible for multilineage engraftment and hematopoietic reconstitution.

15. **Gadbail AR, Korde S, Chaudhary MS, Sarode SC, Gondivkar SM, Dande R, et al. (2020)** compared the expression of *Ki67*, *CD105*, and *α-SMA* between OSCC-OSF and oral squamous cell carcinoma (OSCC). Formalin-fixed paraffin-embedded tissues of 105 OSCC-OSF and 112 OSCC cases were subjected to immunohistochemistry for evaluation of *Ki67*, *CD105*, and *α-SMA* expression where it was found that *Ki67* (labeling index) *LI*, *MVD* and *α-SMA* expression were significantly higher in OSCC compared to OSCC-OSF. *Ki67 LI* and *MVD* was significantly higher in OSCC compared to OSCC-OSF in parameters such as well-differentiated, early TNM stage, non-metastatic, and more than 3-year survival. α-SMA expression was significantly higher in OSCC compared to OSCC-OSF in parameters such as moderate differentiation, metastatic lesions, and survival less than 3 years. *Ki67 LI*, *MVD* and *α-SMA* showed significant positive correlation with each other in OSCC and OSCC-OSF. Therefore, concluding that proliferation, neo angiogenesis and myofibroblast differentiation were significantly higher in the OSCC group compared to the OSCC-OSF group, suggesting the biological distinctness of OSCC-OSF, which could help the future development of targeted therapies.

16. **Jing Y, Zhou Q, Zhu H, Zhang Y, Song Y, Zhang X, et al. (2019)** conducted a study with the aim of evaluation of the prognostic value of Ki-67 in a large number of OSCC patients. Ki-67 expression was detected in 298 OSCC archival tissue specimens and 98 tumor-free oral mucosa specimens (62 dysplasia mucosa and 36 normal mucosa) by immunohistochemical staining. Expression of Ki-67 in normal tissues, dysplasia tissues and OSCC tissues was compared. Associations between Ki-67 expression and clinicopathological parameters were analyzed by $\chi 2$ test. It was observed

that Ki-67 expression was higher in OSCC tissues than in tumor-free tissues and that it exponentially increases with the progression of dysplasia in oral mucosa tissues. Also, patients with increased Ki-67 expression had a worse clinical outcome, including poor tumor differentiation (P=0.001), increased positive lymph node metastasis (P=0.006) and increased worst pattern of invasion type (P<0.0001). Kaplan-Meier survival analysis concluded that higher Ki-67 expression was associated with poorer overall survival (OS) (P=0.035), recurrence-free survival (RFS) (P=0.017), metastasis-free survival (MFS) (P=0.032) and disease-free survival (DFS) (P=0.018) times. Additional multivariate analysis demonstrated that Ki-67 expression was negatively associated with OS, DFS, RFS and MFS. It was found that Ki-67 overexpression is associated with the progression of OSCC and can be used as an independent prognostic factor for OSCC patients.

17. **Schulze AB, Schmidt LH, Heitkötter B, Huss S, Mohr M, Marra A, et al. (2019)** conducted a study on CD34+ and SMA+ CAFs in non-small cell lung cancer (NSCLC) to find out the prognostic and clinical impact. Immunohistochemistry was performed to analyse stromal protein expression of CD34 and SMA in 304 NSCLC patients with pTNM stage I-III disease. The study revealed an association of CD34+ CAFs with G1/2 tumors and adenocarcinoma histology. Also CD34+ CAFs were found to be an independent prognostic factor (both for progression free survival [PFS] and overall survival [OS] in stage I-III NSCLC). SMA+ expression had a correlation with higher pTNM-tumor stages and lymphatic spread (pN stage). SMA-negativity corelated with improved PFS, but no prognostic effect was found on OS. Neither CD34+ CAFs nor SMA+ CAFs were related to the primary tumor size, localization and depth of infiltration (pT stage). Therefore, concluding that CD34 can be used as an independent prognostic marker in pTNM stage I-III NSCLC. Moreover, loss of CD34+ CAFs might effect the dedifferentiation of the NSCLC tumor from its cell origin and SMA+ CAFs were found to be more prevalent in NSCLC tumors of higher stages and lymphonodal positive NSCLC.

18. **El-Kammar, Nermeen S. A, AbdulKhalikD. (2019)** with the aim of evaluation of the potential role of α-SMA in the progression of SCC conducted a study on 24 SCC specimens of different grades. qRT-PCR was performed to evaluate α-SMA gene expression in tongue SCC cell line (SCC 25), normal fibroblasts cell line (Wi-38) and in a coculture of both cells. Results showed that there was α-SMA expression in the epithelial cells and that co-culturing resulted in an overall increase in α-SMA expression. Immunohistochemical evaluation of the specimens revealed that the expression of α-SMA increased with tumor grade and correlated with lymph node involvement. It was concluded that α-SMA can be used as a prognostic marker and a potential target for cancer therapy.

19. **Anggorowati, N., Kurniasari, C.R. and Damayanti, K. et.al. (2017)** analysed the characteristic of α-SMA and collagen in tumor cells and stroma of ovarian neoplasms through their study. They also investigated PCNA (proliferating cell nuclear antigen) expression. Thirty samples including serous, mucinous, endometrioid, and clear cell subtypes were included in the study. Overexpressions of α-SMA in serous and clear cell ovarian carcinoma were detected thus concluding that tumor cells of serous and clear cell ovarian carcinoma show mesenchymal characteristic as seen by by α-SMA positive expression. This expression indicated that the subtypes were more aggressive. This research proved that collagen and α-SMA area fractions in stroma were higher in benign than in malignant neoplasms.

20. **Wang S, Ma N, Kawanishi S, Hiraku Y, Oikawa S, Xie Y, et al. (2014)** In order to investigate the relationships of cancer-associated fibroblasts (CAFs) and chemokine-related molecules with neoangiogenesis, comparison of immunohistochemical analyses of alpha-smooth-muscle actin (α-SMA), stroma-derived factor-1 (SDF-1), and its receptor CXCR4 in primary NPC specimens and chronic nasopharyngitis tissues was done. In addition, the expression of vascular endothelial growth factor (VEGF-A), and CD133- and VEGF- receptor-2 (VEGFR-2) double positive cells, as endothelial progenitor cells (EPCs) were also examined. A significantly

higher expression of α-SMA was observed in fibroblasts in NPC stroma. The immunoreactive intensities of SDF-1 and CXCR4 were significantly higher in NPC cells. CXCR4-positive cells and D133/VEGFR-2- double positive cells were observed in the stroma surrounding cancer nests, and VEGF was detected in both cancer and stromal cells. Microvessel density was significantly higher in the stroma of NPC tissues compared to chronic nasopharyngitis tissues.

21. **Lei Ding, Ziwen Zhang, Duo Shang, Jie Cheng, Hua Yuan, Yunong Wu, Xiaoling Song, Hongbing Jiang (2013)** conducted a study with the aim to investigate the relationship between the expression of myofibroblasts marker, α-SMA, and clinicopathological features, EMT, lymphogenesis, and prognostic status in oral tongue squamous cell carcinoma (OTSCC). Immunohistochemistry was used to detect α-SMA expression in 50 OTSCCs. It was concluded that α-SMA-positive myofibroblasts have important impacts on cancer progression, metastasis, and survival prognosis of patients with OTSCC and the functions of α-SMA-positive myofibroblasts in OTSCC may be associated with promoting EMT of tumor cells and lymphogenesis of metastasis microenvironment.

22. **Pires FR, Ramos AB, Oliveira JB, Tavares AS, Luz PS, Santos TC. (2013)** conducted a study with the aim of reporting demographic and clinicopathological features of OSCC diagnosed in an Oral Pathology service in southeastern Brazil in 8 years time period. OSCC diagnosed from 2005 to 2012 were taken into account and reviewed, with histological analysis of all hematoxylin and eosin stained slides and also demographic and clinical information from the laboratory records. 346 OSCC was retrieved and males represented 67% of the total sample. Mean age of the patients was 62.3 years-old and females were affected a decade older than males ($p<0.001$). Mean time of complaint with the tumors was 10 months and site distribution showed that the border of the tongue (37%), alveolar mucosa/gingiva (20%) and floor of mouth/ventral tongue (19%) were most commonly affected sites. Mean size of the tumor was 3.4 cm, with no differences for males and females ($p=0.091$). Males reported both tobacco

and alcohol consumption more frequently than females. Histological grade of the tumors revealed that 27%, 40% and 21% of the tumors, respectively, were classified as well, moderately and poorly differentiated OSCC, 26 cases (7.5%) were microinvasive OSCC and 17 cases were OSCC variants. OSCC in males mostly affected the border of tongue, floor of mouth/ventral tongue and alveolar mucosa/gingival, while they were more frequent on the border of tongue, alveolar mucosa/gingival and buccal mucosa/buccal sulcus in females (p=0.004). The data in the study reflected that the epidemiological characteristics of OSCC diagnosed in a public Oral Pathology laboratory in southeastern Brazil. The data also highlighted several differences in clinicopathological features when comparing male and female OSCC affected patients.

23. **Vieira SC, Silva BB, Pinto GA, Vassallo J, Moraes NG, Santana JOI, et al. (2005)** conducted a study with the aim to evaluate the association between angiogenesis and the pathoanatomical features of cervical carcinoma using anti-CD34 monoclonal antibody. Sixty-two patients with invasive carcinoma of the uterine cervix in stages Ib-IIa were included. A primary monoclonal antibody specific for CD34 was used. This study suggested that anti-CD34 antibody reactivity in cervical carcinoma is associated with pathoanatomical features indicative of poorer prognosis.

RESEARCH GAPS:

It is well understood that the activity that is coordinated between epithelial cells and their stroma is central in regulating growth and differentiation in normal as well as pathological conditions. Through the progression of the tumor, the stroma imitates disturbed interactions between the neoplastic population and its surroundings. Successful immunotherapy against cancer should essentially include complementary treatments against these tumor-associated fibroblasts. Although studies including α-SMA markers among OSCC patients have been reported, rarely any study involving CD34 marker in OSCC cases have been reported. Also, there are no studies

pertaining to follow-up of the patients to establish the above markers as definite prognostic markers.

Most OSCC patients are also treated with post-operative radiotherapy (PORT) and/or chemotherapy. These treatment modalities have an adverse impact on quality of life. There have been limited advances in OSCC management, with overall survival (OS) improving by only 5% in the last 20 years.

Furthermore, OSCC prognostication and treatment selection still rely heavily on the tumor-node-metastasis (TNM) staging system[2] and Broader`s classification for histological grading; where tumour biology has limited impact on treatment decisions. The most important prognostic factor is the presence or absence of metastases. Invasion and metastasis involve epithelial to mesenchymal transition (EMT). EMT is activated during development and wound healing, however, aberrant activation is associated with cancer progression.5The major role in gross and aggressive behavior of various pathological grades of OSCC is laid by tumoral stroma. Stromal reactions in cancer have a vital diagnostic and prognostic significance. The local aggregation of connective tissue cells and extracellular matrix characterizes epithelial tumors; this phenomenon has been labeled as the stromal reaction. Cells of the stroma have the capability to join actively in the progression of tumor, which in turn allows invasion and metastasis.[1]

Therefore, the current study is undertaken to fill in major research gaps by **evaluating the expression of CD34 and α-SMA and establishing a correlation of these biomarkers (CD34 and α-SMA) in relation to the clinical progression and grading of OSCC** along with the treatment plan followed, thus establishing a definite prognosis by demonstrating the role of CD34 and α-SMA as prognostic markers in oral squamous cell carcinoma.

This will confirm diagnostic and prognostic significance of **CD34** and α-**SMA** and association with tumor differentiation.

AIM:

To establish CD34 and Alpha Smooth Muscle Actin (α-SMA) as diagnostic and prognostic markers in Oral Squamous Cell Carcinoma.

OBJECTIVES:

- To evaluate the expression of angiogenic and myofibroblastic biomarkers in Oral squamous Cell Carcinoma (OSCC)
- To establish a correlation between angiogenic and myofibroblastic biomarkers to the clinical progression and grading of OSCC.
- To associate biomarkers (angiogenic and myofibroblastic) with treatment modality and survival of OSCC patients.
- To establish angiogenic and myofibroblastic as prognostic biomarkers in Oral Squamous Cell Carcinoma.

MATERIALS AND METHOD:

The present study (prospective and retrospective study) will be undertaken in the Department of Oral and Maxillofacial Pathology and Oral Microbiology, Dental College, Swami Vivekanand Subharti University, Meerut to establish angiogenic and myofibroblastic biomarkers as diagnostic and prognostic markers in Oral Squamous Cell Carcinoma after obtaining ethical clearance from the Institutional Research and Development Committee (IRDC) and Institutional Human Ethics Committee (IHEC).

Source of Data

A total number of 180 histopathologically confirmed cases of Oral Squamous Cell Carcinoma (OSCC) with detailed clinical recodes and apparently normal mucosa as control were included in this study. Paraffin embedded tissue blocks of confirmed OSCC cases were retrieved from the archives of the department.

The study groups are as under:

Group A: Well Differentiated Squamous Cell Carcinoma (WDSCC) (n=60)

Group B: Moderately Differentiated Squamous Cell Carcinoma (MDSCC) (n=40)
Group C: Poorly Differentiated Squamous Cell Carcinoma (PDSCC) (n=60)
Group D: Control (Normal Oral Mucosa) (n=10)

Inclusion Criteria:

- Histopathologically diagnosed cases of OSCC (All grades) with detailed clinical records, treatment plan and confirmed demographic details (address and contact numbers)
- Apparently normal mucosa (Control) received during third molar surgical extraction.

Exclusion Criteria:

- Ulcerated/Necrosed epithelium
- Areas of inflammation
- Background or non specific stain (CD34 and α-SMA)

Collection of Data:

For all the groups (n=190) one slide will be stained with H & E, other two slides would be subjected for Immunohistochemical staining with CD34 and α-SMA respectively.

For all the OSCC cases (n=180) detailed case history was recorded in a systematic manner followed by a questionnaire filled by patients.

MATERIALS

1. EQUIPMENTS
    - Semi Automatic Rotary Microtome
    - Hot Plate
    - Hot water bath
    - Pressure cooker/Microwave
    - Pre coated slides with Poly-L-Lysine
    - Staining jars (Coplin Jars)
    - Humidifying chamber
    - Absorbent paper/tissue paper

- Pipettes & Micropipettes
- Cover slips
- Measuring cylinder
- Refrigerator
- Microscope with camera

2. REAGENTS USED FOR HAEMATOXYLIN AND EOSIN STAINING
    - Xylene
    - Ethyl alcohol – 70%, 90%, 100%
    - Counter staining – Harris Haematoxylin
    - 1% acid alcohol
    - Eosin Y

3. REAGENTS USED FOR IHC STAINING
    - Primary antibody for CD34
    - Primary antibody for α-SMA
    - Secondary antibody/detection kit for CD34
    - Secondary antibody/detection kit for α-SMA
    - Blocking agents
    - Super enhancer
    - Chromogen
    - Antigen retrieval: Tris Buffer EDTA pH 7.6
    - Wash Buffer: Tris Buffer saline (TBS) pH 7
    - Counter Stain
    - Xylene
    - Mounting Media: DPX (Dibutylpthalate Polystyrene Xylene)

METHODOLOGY:

Hematoxylin and Eosin (H&E) Staining Protocol

Formalin fixed paraffin embedded tissues will be sectioned at 4-micrometre thickness and would be subjected to routine H&E staining.

Immunohistochemistry Staining Protocol

Principle: IHC staining uses the technique of polymeric labeling. The Super Sensitive Polymer HRP Detection System uses a non-biotin polymeric technology; wherein, the secondary antibody conjugated to Polymer-HRP reagent is bound to the primary antibody and is then visualized by the chromogen.

Tissue preparation and Sectioning: Formalin fixed paraffin embedded tissues will be sectioned at 4 µm and mounted on positively charged slide / Poly-L-Lysine coated slides.

Incubation: Slides will be incubated on slides warming table (Thermostat) for 1 hour.

Deparaffinization:

a) Xylene
   - **a.** Xylene I (5 minutes)
   - **b.** Xylene II (5 minutes)
   - **c.** Xylene III (5 minutes)

Rehydration:

b) Alcohol
   - **a.** 100% (5 minutes)
   - **b.** 85% (5 minutes)
   - **c.** 70% (5 minutes)

c) Will be Washed in running water (5 minutes)

INTERPRETATION AND VALUATION OF CD34 and α-SMA EXPRESSION:

A total number of 180 histopathologically confirmed cases of Oral Squamous Cell Carcinoma (OSCC) and apparently normal oral mucosa will be included in the study. Paraffin-embedded tissue blocks of these confirmed cases will be retrieved from the archives. The study group comprised of **Group A**: Well Differentiated Squamous Cell Carcinoma (WDSCC) (n=60); **Group B**: Moderately Differentiated Squamous Cell Carcinoma (MDSCC) (n=60); **Group C**: Poorly Differentiated Squamous Cell Carcinoma (PDSCC) (n=60); **Group D**: Control (Normal Oral Mucosa) (n=10)

Quantitative analysis:

CD34Positive: CD34 positivity was considered as brown cytoplasmic and/or membrane staining in the endothelial cells of the tumor stroma. A semiquantitative assessment of C34 positivity will be performed wherein the CD34immunostained cells will be evaluated in 5 high power fields for every specimen and expressed as a percentage of positive cells. When stromal cells positive for CD34 was >10%, it was considered positive, and <10% was considered negative for CD34 reaction.

CD34 Negative: Cells with no stain.

α-SMA Positive: α-SMA positivity was considered as brown cytoplasmic and /or membrane staining in stromal cells. A semiquantitative assessment of α-SMA positivity will be performed wherein the α-SMA immunostained cells will be evaluated in 5 high power fields for every specimen and expressed as a percentage of positive cells. When stromal cells positive for CD34 was >10%, it was considered positive, and <10% was considered negative for CD34 reaction.

α-SMA Negative: Cells with no stain.

For each batch of slides, positive and negative controls will run. The tissues will be sectioned (4µm) and stained with CD34 and α-SMA. The stained sections will be scanned under 10x magnification and based on the same cell counting will be performed under 40x magnification in five High Power Fields (HPFs). All stained histopathological slides will be subjected for evaluation by two experienced oral

pathologists using Light microscope and the data will be tabulated in the prescribed Performa.

Qualitative analysis:

The **Staining Intensity** for CD34 as well as α-SMA will be analyzed on the basis of the clarity of stained cells and the scoring will be given as follows

- N: No Expression (no staining)
- 1 = weak expression (pale yellow)
- 2 = intermediate Expression(yellow)
- 3 = strong Expression (dark brown/ dark yellowish brown)

INTERPRETATION AND VALUATION OF CLINICAL RECORDS:

A total number of 120 histopathologically confirmed cases of OSCC (same cases that are used for staining with H &E and IHC CD34 and α-SMA) were followed up for treatment plan and prognosis (via a questionnaire) along with clinical investigations and staging. Clinical details, treatment plan and prognosis were filled in a prescribed Performa.

Statistical Analysis:

Obtained data will be subjected to various comparative and other statistical analysis.

EXPECTED OUTCOME:

A correlation between CD34 and Alpha Smooth Muscle Actin (α-SMA) would be established along with their prognostic significance in Oral Squamous Cell Carcinoma.

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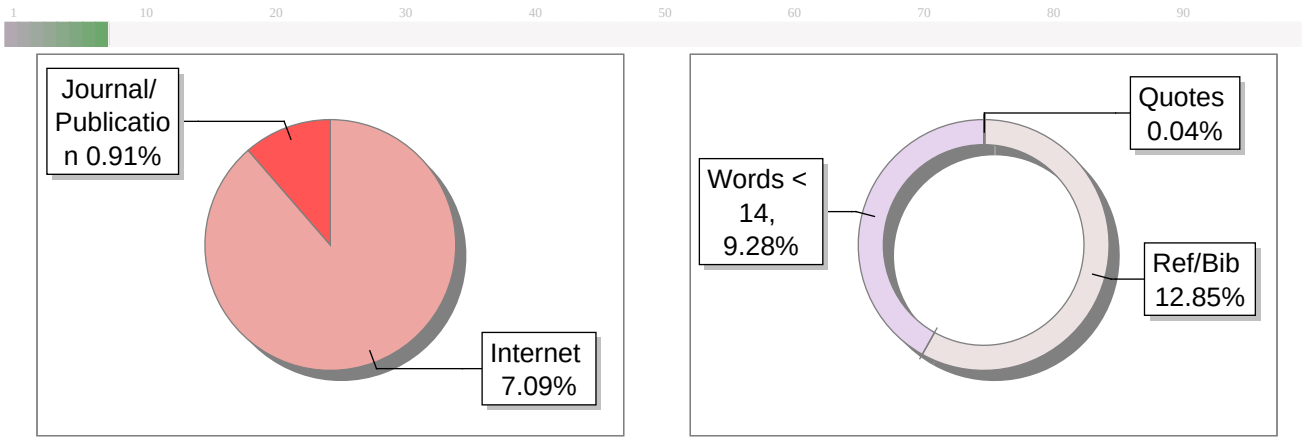

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